

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>5</sup> :</b> C12N 9/08, C11D 3/386, D06L 3/02	<b>A1</b>	<b>(11) International Publication Number:</b> WO 94/12620 <b>(43) International Publication Date:</b> 9 June 1994 (09.06.94)
<b>(21) International Application Number:</b> PCT/DK93/00394 <b>(22) International Filing Date:</b> 1 December 1993 (01.12.93) <b>(30) Priority Data:</b> 1441/92 1 December 1992 (01.12.92) DK <b>(71) Applicant (for all designated States except US):</b> NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsvaerd (DK). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> SCHNEIDER, Palle [DK/DK]; Rydtoften 43, DK-2750 Ballerup (DK). ANDERSEN, Morten, Birket [DK/DK]; Vordingborggade 6F02, DK-2100 Copenhagen Ø (DK). <b>(74) Common Representative:</b> NOVO NORDISK A/S; Patent Department, Novo Allé, DK-2880 Bagsvaerd (DK).		<b>(81) Designated States:</b> BR, CA, FI, JP, KR, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> With international search report.
<b>(54) Title:</b> ENHANCEMENT OF ENZYME REACTIONS  <b>(57) Abstract</b>  This invention relates to activation of enzymes. More specifically, the invention relates to peroxidase enhancing agents. The invention also relates to methods of oxidizing a substrate with a source of hydrogen peroxide in the presence of a peroxidase enzyme and in the presence of a peroxidase enhancing agent. More specifically, the invention relates to a method of bleaching of dye in solutions, to a method of inhibiting the transfer of a textile dye from a dyed fabric to another fabric when said fabrics are washed together in a wash liquor, to a method of bleaching of lignin-containing material, in particular bleaching of pulp for paper production, to a method of treatment of waste water from pulp manufacturing, and to a method of enzymatic polymerization and/or modification of lignin or lignin containing material.		

*FOR THE PURPOSES OF INFORMATION ONLY*

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

## ENHANCEMENT OF ENZYME REACTIONS

### TECHNICAL FIELD

This invention relates to activation of enzymes. More specifically, the invention relates to peroxidase enhancing agents.

The invention also relates to methods of oxidizing a substrate with a source of hydrogen peroxide in the presence of a peroxidase enzyme and a peroxidase enhancing agent. More specifically, the invention relates to a method of bleaching of dye in solutions, to a method of inhibiting the transfer of a textile dye from a dyed fabric to another fabric when said fabrics are washed together in a wash liquor, to a method of bleaching of lignin-containing material, in particular bleaching of pulp for paper production, to a method of treatment of waste water from pulp manufacturing, and to a method of enzymatic polymerization and/or modification of lignin or lignin containing material.

### BACKGROUND ART

Peroxidases (E.C. 1.11.1.7) are enzymes that catalyse the oxidation of a substrate (an electron or hydrogen donor) with hydrogen peroxide. Such enzymes are known from microbial, plant and animal origins, e.g. peroxidase from Coprinus cinereus (cf. e.g. EP 179,486). They are typically hemoproteins, i.e. they contain a heme as a prosthetic group.

Use of peroxidase together with hydrogen peroxide or a hydrogen peroxide precursor has been suggested e.g. in bleaching of pulp for paper production, in treatment of waste water from pulp production, for improved bleaching in laundry detergents, for dye transfer inhibition during laundering, and for lignin modification, e.g. in particle board production.

The compound 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate), ABTS, supplied by Boehringer Mannheim, is a chromogenic substrate, and a common peroxidase and phenol

oxidase assay agent. These enzymes catalyse the oxidation of ABTS by hydrogen peroxide and dioxygen, respectively, producing a greenish-blue colour, which process may be monitored photometrically.

5 ABTS has been found to form a stable radical cation when oxidized by a laccase enzyme (polyphenol oxidase, EC 1.10.3.2), and has been proposed to act as a redox mediator for oxidation of non-phenolic lignin model compounds [Bourbonnais R, Paice M G; FEBS Lett (1990) 267 99-102].

10 Studies on demethylation and delignification of kraft pulp by a laccase enzyme in the presence of ABTS showed that the extent of partial demethylation by laccase was increased in the presence of ABTS [Bourbonnais, R. and Paice, M.G; Appl. Microbiol. Biotechnol. (1992) 36 823-827].

15 Certain oxidizable substrates e.g. metal ions and phenolic compounds such as 7-hydroxycoumarin (7HcM), vanillin (VAN), and p-hydroxybenzenesulfonate (pHBS), have been described as accelerators or enhancers, able to enhance bleaching reactions (cf. e.g. WO 92/18683, WO 92/18687, and Kato M and Shimizu S, Plant Cell Physiol. 1985 26 (7), pp. 1291-1301 (cf. Table 1 in particular), or Saunders B C, et al., Peroxidase, London, 1964, p. 141 ff).

#### SUMMARY OF THE INVENTION

It is an object of the invention to provide an agent for  
25 enhancing the activity of peroxidase enzymes, and to provide a method of enhancing the activity of peroxidase enzymes. It has now surprisingly been found that the activity of peroxidases increases significantly in the presence of an azino compound as described herein.

30 Accordingly, in its first aspect, the present invention provides an agent for enhancing the activity of a peroxidase enzyme, the agent being described by the general formula



oxidase assay agent. These enzymes catalyse the oxidation of ABTS by hydrogen peroxide and dioxygen, respectively, producing a greenish-blue colour, which process may be monitored photometrically.

- 5 ABTS has been found to form a stable radical cation when oxidized by a laccase enzyme (polyphenol oxidase, EC 1.10.3.2), and has been proposed to act as a redox mediator for oxidation of non-phenolic lignin model compounds [Bourbonnais R, Paice M G; FEBS Lett (1990) 267 99-102].
- 10 Studies on demethylation and delignification of kraft pulp by a laccase enzyme in the presence of ABTS showed that the extent of partial demethylation by laccase was increased in the presence of ABTS [Bourbonnais, R. and Paice, M.G; Appl. Microbiol. Biotechnol. (1992) 36 823-827].
- 15 Certain oxidizable substrates e.g. metal ions and phenolic compounds such as 7-hydroxycoumarin (7HCm), vanillin (VAN), and p-hydroxybenzenesulfonate (pHBS), have been described as accelerators or enhancers, able to enhance bleaching reactions (cf. e.g. WO 92/18683, WO 92/18687, and Kato M and
- 20 Shimizu S, Plant Cell Physiol. 1985 26 (7), pp. 1291-1301 (cf. Table 1 in particular), or Saunders B C, et al., Peroxidase, London, 1964, p. 141 ff).

#### SUMMARY OF THE INVENTION

It is an object of the invention to provide an agent for  
25 enhancing the activity of peroxidase enzymes, and to provide a method of enhancing the activity of peroxidase enzymes. It has now surprisingly been found that the activity of peroxidases increases significantly in the presence of an azino compound as described herein.

- 30 Accordingly, in its first aspect, the present invention provides an agent for enhancing the activity of a peroxidase enzyme, the agent being described by the general formula



in which formula A and B, which may be identical or different, independently represents any of the substituents presented in Fig. 1;

in which substituents the symbols X and Y, which may be 5 identical or different, independently represents carbon, nitrogen, which nitrogen may be unsubstituted or substituted with a substituent group  $R^5$ , sulfur, oxygen, selenium or tellurium;

and in which substituents the substituent groups  $R^1$ ,  $R^2$ , 10  $R^3$ , and  $R^4$ , which may be identical or different, independently represents hydrogen, halogen, a hydroxy group, a  $C_1$ - $C_3$  alkoxy group, a formyl group, a carboxy group, a sulfo group, a nitro group, a  $C_1$ - $C_5$  alkyl group, which alkyl group may furthermore be saturated or unsaturated, or an amino group, which amino group 15 may furthermore be unsubstituted or substituted once or twice with a substituent group  $R^5$ ;

which substituent group  $R^5$  represents halogen, a hydroxy group, a  $C_1$ - $C_3$  alkoxy group, a  $C_1$ - $C_5$  alkyl group, or an amino group.

20 In its second aspect, the invention provides a method of oxidizing a substrate with a peroxidase enzyme, in the presence of a source of hydrogen peroxide, and in the presence of a peroxidase enhancing agent of the invention.

In a specific aspect, the invention provides a method of 25 inhibiting the transfer of a textile dye from a dyed fabric to another fabric when said fabrics are washed together in a wash liquor, the method comprising treatment of the wash liquor with a peroxidase enzyme in the presence of a source of hydrogen peroxide and in presence of a peroxidase enhancing agent of the 30 invention.

In a particular aspect, the invention provides a detergent additive capable of inhibiting the transfer of a textile dye from a dyed fabric to another fabric when said fabrics are washed together in a wash liquor, the detergent 35 additive comprising an enzyme exhibiting peroxidase activity, a source of hydrogen peroxide and a peroxidase enhancing agent of the invention.

in which formula A and B, which may be identical or different, independently represents any of the substituents presented in Fig. 1;

in which substituents the symbols X and Y, which may be 5 identical or different, independently represents carbon, nitrogen, which nitrogen may be unsubstituted or substituted with a substituent group  $R^5$ , sulfur, oxygen, selenium or tellurium;

and in which substituents the substituent groups  $R^1$ ,  $R^2$ , 10  $R^3$ , and  $R^4$ , which may be identical or different, independently represents hydrogen, halogen, a hydroxy group, a  $C_1$ - $C_3$  alkoxy group, a formyl group, a carboxy group, a sulfo group, a nitro group, a  $C_1$ - $C_5$  alkyl group, which alkyl group may furthermore be saturated or unsaturated, or an amino group, which amino group 15 may furthermore be unsubstituted or substituted once or twice with a substituent group  $R^5$ ;

which substituent group  $R^5$  represents halogen, a hydroxy group, a  $C_1$ - $C_3$  alkoxy group, a  $C_1$ - $C_5$  alkyl group, or an amino group.

20 In its second aspect, the invention provides a method of oxidizing a substrate with a peroxidase enzyme, in the presence of a source of hydrogen peroxide, and in the presence of a peroxidase enhancing agent of the invention.

In a specific aspect, the invention provides a method of 25 inhibiting the transfer of a textile dye from a dyed fabric to another fabric when said fabrics are washed together in a wash liquor, the method comprising treatment of the wash liquor with a peroxidase enzyme in the presence of a source of hydrogen peroxide and in presence of a peroxidase enhancing agent of the 30 invention.

In a particular aspect, the invention provides a detergent additive capable of inhibiting the transfer of a textile dye from a dyed fabric to another fabric when said fabrics are washed together in a wash liquor, the detergent 35 additive comprising an enzyme exhibiting peroxidase activity, a source of hydrogen peroxide and a peroxidase enhancing agent of the invention.